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Pathogenicity of Isolates of *Metarhizium anisopliae* from Gujranwala (Pakistan) against *Coptotermes heimi* (Wasmann) (Isoptera: Rhinotermitidae)

SOHAIL AHMED[1], MUHAMMAD RIZWAN ASHRAF, ABID HUSSAIN AND MUHAMMAD ASAM RIAZ
Department of Agri. Entomology, University of Agriculture, Faisalabad, Pakistan
[1]Corresponding author's e-mail: saha786_pk@yahoo.com

ABSTRACT

Three isolates/strains of *Metarhizium anisopliae* were isolated from swarmed adults of *Coptotermes heimi* at No-shehra Virkan, Gujranwala. The exposure methods of these isolates against termites included soil substrate and filter paper treatment with different concentrations (1×10^4, 1×10^6, 1×10^8, 1×10^{10} conidia mL^{-1}) of conidial suspension. LT_{50} for three strains of *M anisopliae* on *C. heimi* were comparatively more (65-106 h) in soil than on filter paper (50-83 h). There was significant difference in different concentrations of all strains of *M. anisolpliae* ($p<0.05$).

Key Words: Fungal pathogen; Termites; *Coptotermes heimi*; Direct exposure

INTRODUCTION

Entomopathogenic fungi are potentially the most useful against many species of the termites (Milner & Staples, 1996; Milner *et al*., 1998; Milner *et al*., 2003), which may be applied in different application methods such as direct exposure, soil barrier and in baits. The genus *Metarhizium*, *Beauveria* and *Paecilomyces* are fungal pathogens of insects that have shown great promise in commercial development. *Metarhizium anisopliae*, with worldwide distribution has been isolated from more than 200 insect species across seven orders, has shown great potential as a bio-control agent and is under development commercially in several countries as a control agent against many species. Strains of *Metarhizium* differ in their host range, necessitating selection of the most virulent strain against a target insect (Zimmerman, 1993). *M. anisopliae* has an advantage over *Beauveria bassiana* in termite microbial control due to termite social behaviour and high total fungal biomass production as compared to *Beauveria bassiana* (Sun *et al.*, 2002), Many strains of *M. ansisopliae* have been isolated from termites and are reported elsewhere (Lai *et al.*, 1982; Wells *et al.*, 1995; Zoberi, 1995; Liu *et al.*, 2002; Sun *et al.*, 2003; Wright *et al.*, 2005).

There is no record of such behaviour of locally available *M. ansiopliae* strains against the termites in Pakistan. Hussain (2006) tested exotic strains (ARSEF 6911 & ARSEF) and found that direct exposure of fungal spores to termites caused lower LT_{50} values as compared to surface crawling and substrate treatment. Two local strains of *M. anisopliae* were isolated from swarming adults of *Coptotermes heimi* from Faisalabad and Murree, Pakistan. Three strains of *M. anisopliae* collected from termites in Murree (Pakistan) has as similar characteristics of virulence against *Coptotermes heimi* as were previously reported from those strains at Faisalabad, Pakistan, however, LT_{50} for Murree were comparatively more (31-69 h) in filter paper than in soil (49-131 h) as compared to (91-135 h) in filter paper and (93-175) in soil of the strains from Faisalabad (Ahmed *et al*., 2008 a & b).

In this paper isolation and pathogenicity of three strains of *M. anisopliae* from swarming alates of *Coptotermes heimi* (Was.) (Rhinotermitidae: Isoptera) from No-Shehra Virkan, District Gujranwala, Pakistan is being reported. Noshehra Virkan, Gujranwala is rice growing area in Punjab Province, Pakistan. The termite species, *C. heimi*, along with *Odontotermes* and *Microtermes* has been reported to be abundant in various places in the province (Ahmed *et al.*, 2006, 2007).

MATERIALS AND METHODS

The swarmed alates were collected alive and brought to the Termites Management Laboratory, Department of Agri. Entomology, University of Agriculture, Faisalabad, Pakistan.

Fungus isolation. Fungi were isolated by using three methods:

1) Isolation from alates. In this method, alates after swarming were collected and transferred to the petri dishes (100 × 15 mm) containing 50 g sterilized soil. As soon as fungus appeared on the body of the alates, they were removed from Petri dishes and put in a separate Petri dish till appearance of the spores, which were then inoculated on

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the Potato Dextrose Agar media (PDA) for further propagation of the fungal spores for identification and pathogenicity trials of the isolates.

2) Filter paper isolation method. Fifty alates were released to the petri dishes (100 × 15 mm) containing filter paper in each. The filter paper was moistened with sterilized distilled water. The method was used to determine whether alates were not infected with fungi from the soil or alates carried fungus inside them. The fungus appeared on the body of the dead alates due to the pathogencity of the fungus. The spores of the fungus were inoculated on the PDA media for further propagation, identification and pathogenicity trials of the isolates.

3) Decontamination termite isolation method. A piece of wet filter paper (100 mm diameter) was placed on top of the soil for 10-20 min in a Petri dish containing 40 termite workers. The termites trapped in the filter paper then were transferred into an empty Petri dish (100 × 15 mm) for surface decontamination of the termites, which was essential for removal of saprophytes on the body surface. 40 termites from each test jar were placed on a sieve (30 mesh) fitted in a lid and placed in 1% $NaClO_4$, plus 0.005% of Tween 80, for 1 min and then they were briefly rinsed in 2-3 changes of sterile water. All washed termites were put on sterilized filter paper to dry and were transferred to four Petri dishes (100 × 15 mm), 25 termites/dish, with a wet piece of filter paper in each dish as the source of food and moisture. Petri dishes were incubated at 27°C without light for up to 12 days and mortality was recorded daily. Dead termites were surface decontaminated as above, except that they also were immersed in 70% alcohol for 5 sec before NaClO treatment. The termites then were placed on potato dextrose agar (PDA) medium and incubated up to 12 days at 27°C. The conidiogenesis was observed; the fungus was transferred onto PDA (Smith, 1993).

Propagation of fungal strains. Strains of fungi were maintained on PDA media, which was prepared by taking 20 g of agar and 20 g of glucose and mix in 500 mL water in a conical flask by shaking simply. 200 g of peeled potatoes into pieces were boiled in 500 water until potatoes become soft. The potatoes extract was poured into a 1000 mL conical flask after straining and volume was made up to 1000 mL. This medium was poured in test tubes in slants and these tubes were then tightly plugged with cotton. Test tubes were sterilized at 121°C for 15 min. After cooling, these slants were inoculated with spores in laminar airflow cabinet. These slants were placed in an incubator at 28 ± 2°C for 7 days. After 3-4 days white color mycelium was observed, which turned green on sporulation then these were stored at 4°C.

Collection of termites. The workers of *C. heimi* were collected from Residential Halls in the campus of University of Agriculture, Faisalabad. Corrugated card boards were inserted into the holes in cupboard created by activation of *C. heimi*. These card boards were brought in the laboratory and kept in a glass jar containing moistened corrugated card board sheets. From these collections, workers at least 3rd instar (as determined by size) was sorted out within 3 days of the beginning of the experiment.

Preparation of solutions. Spores of different strains of entomopathogenic fungi were harvested from 25-30 days old culture by introducing 1 mL of 0.03% solution of Tween 80 (polyoxyethylene sorbitan monolaurate) in test tubes with slants. These tubes were shaken gently to separate the spores from culture. The spore suspension was decanted into a measuring cylinder (5 mL). The 20 µL of this spore suspension was put on the stage of Neybauer Haemocytometer and concentration of spore was determined. Different concentrations of spores were prepared by diluting the stock solution with 0.03% Tween 80.

Bioassay. Bioassay was conducted by using the following method:

In this experiment the termites were treated with the spore suspension of the isolates of *M. anisopliae* that was isolated from the different localities of Gujranwala (Ga1, Ga3 & Ga4). In this experiment the termites were exposed to the spore suspension of these isolates with the help of Burkard Micro Applicator. The different concentrations for spore suspension were C_1 (1×10^{10}), C_2 (1×10^8), C_3 (1×10^6), C_4 (1×10^4) and C_5 (control). The data was recorded firstly after every 4 h and then after every 12 h.

Observation of fungi on cadavers. At each observation dead termites were counted, removed from the Petri dishes and transferred to another Petri dish containing Potato Dextrose Agar to allow propagation of fungi associated with the cadaver.

Dose mortality studies were conducted by using spore suspensions ranging from 1×10^{10} to 1×10^4, spores/mL spore/mL from data obtained from above mentioned experiments. Data were recorded up to 100% mortality and Lt_{50} values were calculated through Probit analysis. Comparative data on time to death were analyzed as estimates of Lethal Time (LT_{50}), which is the number of days to achieve 50% mortality.

RESULTS

A- Termites treated with spore suspension and put on filter paper. Table I shows the lethal time (LT_{50}) of three isolates of *M. anisopliae* against *C. heimi* when latter were treated with different concentrations of spore suspension of *M. anisopliae* isolates (Ga1, Ga3 & Ga4) and put on the filter paper. Overall, the concentrations of isolate Ga4 with LT_{50} ranging from 81.40 to 50.57 h performed better as compared to Ga1. Ga3, which showed high LT_{50} values except at concentration C4, which was less than that in Ga1 and Ga 4. Slope values were high in the presence of spore as compared to control treatment showing mortality of termites at distinct regular intervals in the control. The heterogeneity (H) were 5.87, 26.08, 4.52, 7.25 and 21.89 for Ga1, where as 40.44, 10.86, 4.93, 1.94 and 21.89 in case of Ga3 and 13.38, 4.31, 3.16, 7.56 and 21.89 in case of Ga4 for C_1 (1 x

Table I. LT_{50} values of different concentrations of *M. anisopliae* (Isolates Ga1, Ga3, Ga4) for *Coptotermes heimi* in treated filter paper

Isolates	Conc.	LT_{50} Hrs.	Slope ± SE	FL (95%)	Fit of Probit line		
					$\chi 2$	g	H
Ga1	C_1 (1 x 10^{10})	54.07	4.954±.275	41.5 - 56.5	46.958	0.096	5.87
	C_2 (1 x 10^8)	60.81	5.042±.280	38.6 - 81.7	208.61	0.429	26.08
	C_3 (1 x 10^6)	62.07	4.256±.240	53.4 - 59.7	36.140	0.161	4.52
	C_4 (1 x 10^4)	83.54	4.815±.247	73.4 – 95.3	79.765	0.092	7.25
Ga3	C_1 (1 x 10^{10})	58.29	5.050±.303	47.2 - 75.6	28.310	0.814	40.44
	C_2 (1 x 10^8)	62.04	6.005±.326	63.7 - 87.8	86.953	0.170	10.86
	C_3 (1 x 10^6)	74.86	6.933±.397	74.9 - 83.9	15.489	0.134	4.94
	C_4 (1 x 10^4)	79.25	5.953±.397	74.8 - 85.6	21.589	0.034	1.93
Ga4	C_1 (1 x 10^{10})	50.57	4.965±.290	39.9 - 66.4	93.678	0.255	13.38
	C_2 (1 x 10^8)	53.88	4.419±.248	45.9 - 60.2	34.473	0.072	4.31
	C_3 (1 x 10^6)	64.67	5.501±.304	56.4 – 78.2	1213.0	0.091	3.16
	C_4 (1 x 10^4)	81.40	4.901±.239	74.3 – 86.7	83.214	0.875	7.56
	C_5 (Control)	416.55	1.790±.179	498.8-907.2	51.182	0.039	20.53

Conc. Concentrations; FL, Fiducial limits

Table II. LT_{50} values of different concentrations of *M. anisopliae* (Isolates Ga1, Ga3, Ga4) for *Coptotermes heimi* in treated soil

M. anisopliae Isolates	Conc.	LT_{50} Hrs.	Slope ± SE	FL (95%)	Fit of Probit line		
					$\chi 2$	g	H
Ga1	C_1 (1 x 10^{10})	66.35	5.211±.284	60.7 -71.7	20.471	.041	2.56
	C_2 (1 x 10^8)	73.85	4.639±.228	45.6 - 109.1	44.743	.477	4.06
	C_3 (1 x 10^6)	89.03	5.620±.264	85.6 - 92.9	14.122	.011	1.086
	C_4 (1 x 10^4)	114.11	4.737±.222	103.1-115.5	25.112	.020	1.93
Ga3	C_1 (1 x 10^{10})	65.69	7.015±.382	60.5 - 70.1	22.350	.044	2.79
	C_2 (1 x 10^8)	84.10	5.836±.298	79.2 - 9.0	19.810	.026	1.98
	C_3 (1 x 10^6)	89.28	5.176±.267	89.2 - 104.5	37.904	.045	3.45
	C_4 (1 x 10^4)	103.44	4.924±.227	87.5 -102.8	47.616	.036	3.66
Ga4	C_1 (1 x 10^{10})	70.22	4.326±.250	61.1 - 77.9	33.956	.075	4.24
	C_2 (1 x 10^8)	68.58	4.070±.225	61.2 - 75.3	27.609	.048	3.07
	C_3 (1 x 10^6)	97.20	4.780±.237	78.8 - 92.7	33.800	.036	3.07
	C_4 (1 x 10^4)	106.72	5.033±.251	93.9 - 119.9	97.310	.096	8.11
	C_5 (Control)	511.90	5.965±.290	468.8-991.5	424.39	0.059	21.89

Conc. Concentrations; FL, Fiducial limits

10^{10}), C_2 (1 x 10^8), C_3 (1 x 10^6), C_4 (1 x 10^4) and C_5 (control), respectively. The g value for all the concentrations were between 0.01-0.8 in all the isolates of *M. anisopliae*.

B-Termites treated with spore suspension and put on soil. Table II shows the lethal time (LT_{50}) of three isolates of *M. anisopliae* against *C. heimi* when latter were treated with different concentrations of spore suspension *M. anisopliae* isolates (Ga1, Ga3 & Ga4) and were put in the soil. Overall, the concentrations of isolate Ga4 performed better as compared to Ga1 and Ga3 by showing low LT_{50} values. Slope values had non-significant difference among the levels.

The analysis of variance of LT_{50} of different *M. anisopliae* isolates at different spore concentrations showed that there was a highly significant difference in the means of different concentrations of spore suspension of *M. anisopliae*. C1 and C4 had significant difference between each other. However, isolates and interaction of isolates and concentration had non-significant different among them (Table III).

Table III. Comparison of LT_{50s} in different concentrations of spore suspension of *M. aniosopliae* isolates on filter paper and in the soil, separately

	Filter paper	Soil
C1	54.31 D	67.42 D
C2	58.91 CD	75.51 D
C3	67.21 C	91.84 C
C4	81.98 B	108.09 B
C5	416.55 A	511.9 A
LSD	9.738	11.28

Any two means not sharing a letter in a column differ significantly at 5% level of probability

DISCUSSION

Three strains of *M. anisopliae* collected from termites in Gujranwala were different from previously repotted isolates of this fungus from *C. heimi* at Murree and Faisalabad (Pakistan). Strains collected from latter places had significant difference not only among them but also their interaction with different concentrations as well (Ahmed *et al*., 2008 a & b). Strains from Gujranwala exhibited non-significant difference of virulence to *C. heimi*. Interaction of strains and concentrations was also non-significant.

The reasons for difference in virulence of isolates/strains have been attributed to many factors. High relative humidity inside a subterranean termite colony and a

confined area are considered conducive to the growth and survival of pathogenic agents such as fungi (Ignoffo, 1992). Growth rates over a range of temperatures (notably growth at temperature above 30ºC); posing no health issues for humans or higher animals; ease and cost of mass production; robustness of spores permitting easy formulation and storage; and longevity of spores, have to be investigated before an isolate could be taken into the field (Milner & Staples, 1996; Milner *et al.,* 1998). The isolates differed in their inoculum potential, rate of mortality and response by other workers to fungus-killed cadavers (Jones *et al.*, 1996). The potential use of *M. anisopliae* as a biological control agent against subterranean termites is being challenged, because the disease remained at an enzootic level in laboratory sand arenas and the fungus showed a reduced survival rate in the presence of termites (Chouvenc *et al.*, 2008). Under laboratory conditions, manipulation of the insects and abiotic conditions during the setting up and running of the bioassays; it is therefore fairly variable and may not represent the real virulence of the pathogen. A possible cause for the differences in strain virulence may be germination speed and consequent penetration (Neves & Alves, 2000). *Metarhizium* isolates with less repellent conidia are as a rule, less virulent and may not be as effective in causing an epizootic in the field (Milner, 2003).

A possible source of variation among the strains of this soil fungus could be difference in soil of different places and ability of strains to survive under different climatic condition and edaphically. Though growth of this fungus has not been studies in this context but Gujranwala strain was collected from soil, where high soil moisture level prevails and this may enhance the ability of the fungi to attach to the termites as has been reported by Ignoffo (1992). Virulence of pathogen may also depend upon the media on which it is being cultured. The comparative growth and pathogencity of the strains will be determined as further step in order to find the difference among the strains.

The LT_{50} values exhibited a dosage dependent mortality both in soil and filter paper substrates. Rosengaus *et al.* (1999) observed that higher spore concentration of *M. anisopliae* (2.2×10^8) spores/mL was more lethal to the termite population. A significant survival in some of the nymphs was observed for longer durations when subjected to lower concentration (10^6 spores mL^{-1}) in the present study. These results are also in confirmation with the work of Rosengaus and Traniello (1997), Liu *et al.* (2002) and Wright *et al.* (2005), who reported that the susceptibility of the termites to fungal infection was often dose dependent.

Based on present results, it is clear that *M. anisopliae* strain had a significant and deleterious effect on the Coptotermes heimi workers. Recognition of right strain after looking at the right set of virulence (exposure time & dose) would be useful in improving the performance of *M. anisopliae* in the field.

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